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Antipeptide Antibodies to the β_2-Adrenergic Receptor Confirm the Extracellular Orientation of the Amino-Terminus and the Putative First Extracellular Loop

Magali A. Théveniau†, John R. Raymond‡, and Geneviève N. Rougon†
†URA 202 CNRS, Institut de Chimie Biologique, Université de Provence, 3 Place V. Hugo, Marseille 1331 Cedex 3 France, and
‡Department of Internal Medicine (Nephrology Division) and Howard Hughes Medical Institute, Duke University Medical Center, Durham, North Carolina 27710

Summary. We developed site-directed rabbit antisera against synthetic peptides selected from the deduced amino acid sequence of the hamster lung β_2-adrenergic receptor (amino acids 16–31 and 174–189, respectively). All antisera directed against peptide 1 (four of four rabbits) as well as two antisera directed against peptide 2 (two of four rabbits) recognized the purified β_2-adrenergic receptor in immunoblot conditions when used at a dilution of 1 : 500. Antisera directed against peptide 1 as well as peptide 2 were able to immunoprecipitate iodinated as well as ^{125}I-cyanopindolol labeled β_2-adrenergic receptor. This last result implies that the recognized epitopes do not contain the ^{125}I-cyanopindolol binding domain of the β_2-adrenergic receptor. Immunoblot experiments performed on membrane fractions from hamster lung tissue showed that immunoreactive bands at 64,000, 57,000, 47,000, 44,000 and 38,000 daltons were specifically detected. When purified β_2-adrenergic receptor was iodinated and submitted to glycolytic and/or tryptic treatments, species with similar molecular weights could be recovered. Then, the immunoreactive bands probably correspond to native β_2-adrenergic receptor and to degradative or nonglycosylated species of this molecule. The antisera were also able to detect immunoreactive molecules in murine and human cell lines, suggesting conservation of the probed sequences between these species. Enzymatic linked immunosorbent assay tests on intact cells and immunofluorescence studies confirmed that the amino-terminus and putative first extracellular loop are extracellularly located. Immunofluorescence studies on mouse brain primary cultures showed that cells expressing β_2-adrenergic receptor-like molecules exhibited a neuronal phenotype.

Key Words β-receptor · antipeptide antibodies · immunoprecipitation · immunoblotting · membrane organization

Introduction

The β_2-adrenergic receptor (β-AR) is a member of a large family of membrane-bound hormone receptors involved in guanine nucleotide regulatory (G-protein)-mediated signaling [11]. In the presence of agonist, β-AR stimulates adenylate cyclase through the G-protein: Gs [11, 35]. In a similar fashion, hormone receptors are linked through a variety of G-proteins to other cellular events such as opening of ion channels [5, 34], activation of cGMP phosphodiesterase [42] or phospholipase C [43], or inhibition of adenylate cyclase [30]. Amino acid sequence information from molecular cloning of β-AR [9, 13, 17, 22, 49], rhodopsin [33], M_1 [25] and M_2 receptors [26] and a protein termed G21 identified as the 5-HT_{1A} receptor [14, 23] demonstrates considerable homology in transmembrane regions of the various receptors [29].

On the basis of structural and functional similarities between β-AR and rhodopsin Dixon et al. [9] have proposed a model in which the β-AR resides in the membrane in a conformation in which the polypeptide chain crosses the lipid bilayer seven times; these putative transmembrane regions are arranged as hydrophobic alpha helices. The model also proposes that the glycosylated amino-terminus is oriented towards the extracellular surface, and that the carboxyl-terminus is cytoplasmic. These features provide a basis for a testable hypothesis about how the β-AR structure determines its function. To date, except for the work of Dohlman et al. [10], there have been few specific tests of any aspect of this model. Antipeptide antibodies have been utilized in a number of laboratories to map functions of specific protein domains based on immunochemical recognition [*see* 31, 41 and 45 for review]. In this work, we used this approach to test one aspect of the validity of the model proposed for β-AR. Antibodies against two synthetic peptides based on residues 16–31 and 174–189 of the hamster β_2-AR [9] were utilized to determine the location of these sequences with respect to the plane of the membrane bilayer in tissues and cell lines of different origin.

Materials and Methods

Reagents

Amino acids were purchased from Beckman (Palo Alto, CA) and Bachem (Bubendorf, Switzerland). Pure rhodopsin was a kind gift from Dr. Hé (CIML, Marseille). Goat antirabbit peroxydase and trypsin, were purchased from Sigma (France); endoglycosidase F from New England Nuclear (Boston, MA).

Peptide Synthesis

Peptides corresponding to residues 16–31 (peptide 1) and 174–189 (peptide 2) of the hamster β_2-AR and analogues with an additional tyrosine were manually assembled from protected amino acids by the solid phase method [1]. The side chains of Asp, Ser and Thr were protected by benzyl groups and the phenol of Tyr by 2,6-dichlorobenzyl. Deprotection and cleavage was performed with liquid hydrogen fluoride containing 10% (vol/vol) anisole for 1 hr, at 4°C. Cysteine residues of peptide 2 were reduced with tributylphosphine and pyridilated with 2,2′ dipyridyldisulfide. Peptides were then purified by gel filtration on Biogel P2 in 0.2 M acetic acid. Purity was assessed by high performance liquid chromatography on a C18 column using gradients of acetonitrile in 0.1% (vol/vol) trifluoroacetic acid and by amino analysis after hydrolysis in 6N HCl for 20 hr. Peptides were at least 90% pure and were used without subsequent purification.

Production of Antibodies

Peptides were coupled to keyhole limpet hemocyanin (KLH) with either glutaraldehyde or N-ethyl, N′ dimethylaminopropyl carbodiimide (ECCD) as described previously by Rougon et al. [36, 38]. In order to orient the coupling of peptide 1 towards the carboxyl-terminus, amino, hydroxy and sulfhydryl groups were protected during coupling reactions by a reversible citraconic acid modification [8]. Coupling efficiency was estimated by recovery of trace amounts of ^{125}I-labeled peptides added to the reaction.

New Zeland white rabbits received subcutaneous injections of the immunogens (1 mg) emulsified with Freund's complete adjuvant. Booster injections were administered at four-week intervals, and rabbits were bled 10–14 days after injection. Antisera were recovered and named according to the rabbit number. Peptide 1: rabbits 1–4 (for rabbits 1 and 2, peptides were citraconylated before linkage to carrier with ECCD); peptide 2: rabbits 5–8 (rabbits 5 and 8 were immunized with glutaraldehyde-linked peptide while rabbits 6 and 7 received ECCD-linked peptide).

Radioimmunoassay

Peptides were iodinated according to the procedure of Salacinski et al. [39]. Antisera were tested for their ability to react with peptides as previously described [20, 36, 38]. Competition liquid phase radioimmunoassays were performed by incubation of the unlabeled peptides with the chosen dilution of antiserum and iodinated peptides. The titer of an antiserum was defined as the dilution required to bind 50% of the radioactivity in the assay.

β_2-AR Purification and Immunoprecipitation

Hamster lung membranes were purified by alprenolol Sepharose affinity chromatography [3], and iodinated by Chloramine T reaction [21]. For immunoprecipitation, the purified β_2-AR was added to 100 μl of buffer per condition to yield a final concentration of 1.5% Triton X100, 0.15% SDS and 1 mM EDTA in PBS. For preclearing, the β_2-AR was incubated with 5 μl preimmune serum for 1 hr at room temperature on a rotator, then with 30 μl of 50% ProtA Sepharose (preblocked with alprenolol chromatography pass through fraction). The precleared supernatant was harvested by centrifugation at 14,000 rpm for 2 min in an Eppendorf micro-centrifuge. Then 7 μl of crude antipeptide antisera was added and the mixture was rotated overnight in the cold room. The mixture was centrifuged as above, the supernatant was discarded and the beads were washed by resuspension in 1 ml of the reaction buffer followed by centrifugation. The wash step was repeated three times. The immunoprecipitated β_2-AR was harvested from the beads by vigorous vortexing in 100 μl of electrophoresis sample buffer at room temperature. The mixture was centrifuged, and 50 μl of each sample were loaded onto 10% polyacrylamide gels for electrophoresis.

β_2-AR Enzymatic Treatments

^{125}I-labeled β_2-AR (10^6 cpm), in 100 μl of 25 mM Tris-HCl, pH 7.4, and 0.05% digitonin were treated with 10^{-2} unit of TPCK-treated trypsin for 2 min at 24°C. Reaction was stopped by boiling in sample buffer. This reaction was also conducted subsequently to endoglycosidase F treatment.

For endogylcosidase F treatment, samples of 10^6 cpm of ^{125}I-labeled β_2-AR in 0.1 M phosphate buffer, pH 6.9, 5 mM EDTA, 1% β-mercaptoethanol, 1% Nonidet P40 were incubated with 0.1 unit of enzyme for 4 and 8 hr, respectively, at 37°C. Products resulting from the digestions were analyzed on 10 or 12% SDS polyacrylamide gel. Gels were dried and subjected to autoradiography.

Membrane Preparation

Tissues or cells were disrupted in 5 volumes (wt/vol) of 10 mM Tris-HCl, pH 7.5, 0.32 M sucrose, 3 mM $MgCl_2$ containing 5 mM iodoacetamide, 20 IU/ml aprotinin, 0.1 μM leupeptin, 1 μM α2-macroglobulin, 0.1 mM phenylmethylsulfonyl fluoride as protease inhibitors and 0.02% sodium azide. Homogenates were centrifuged at 3,000 × g for 15 min. The supernatant was collected and centrifuged at 100,000 × g for 90 min. The resulting pellet was suspended in 10 mM Tris-HCl, pH 8 containing 2% deoxycholate and the above mentioned protease inhibitors. After one cycle of freezing and thawing, the suspension was centrifuged at 100,000 × g for 60 min and the supernatant collected. All procedures were done at 4°C.

Immunoblotting

Proteins were measured by a modification of the method of Lowry [12]. Samples were diluted to 10 mg/ml by mixing with electrophoresis sample buffer containing 5.6% SDS and 10% β-mercaptoethanol and heated at 90°C for 3 min. Proteins were separated according to Laemmli [28] on 10% SDS-polyacrylamide gels and blotted onto nitrocellulose nitrate sheets (0.45 μm

Table. Deduced amino acid sequences of the peptides 1 and 2[a]

PEPTIDE 1

	16 … 31
HAMSTER βAR:	G S H V P D H D V T E E R D E A
HUMAN βAR:	R S H A P D H D V T Q Q R D E V
TURKEY βAR:	A P T G S R Q V S A E L L S Q Q
BOVINE RHODOPSIN:	V R S P F E A P Q Y Y L A E P W
CEREBRAL MUSCARINIC REC.:	A V S P N I T V L A P G K G P W
CARDIAC MUSCARINIC REC.:	N S S N S G L A L I S P Y K T F

PEPTIDE 2

	174 … 189
HAMSTER βAR:	Y R A T H Q K A I D C Y H K E T
HUMAN βAR:	Y R A T H Q E A I N C Y A N E T
TURKEY βAR:	W R D E D P Q A L K C Y Q P D G
BOVINE RHODOPSIN:	R Y I P E G M Q C S C G I D Y Y
CEREBRAL MUSCARINIC REC.:	Y L V G E R T V L A G Q C Y I Q
CARDIAC MUSCARINIC REC.:	F I V G V R T V E D G E C Y I Q

From Dohlman et al. [11]

[a] Homologies between hamster, human and avian sequences for β_2-AR and other related receptors.

pore size) by transverse electrophoresis. Antibody staining was performed at dilution 1 : 500 as previously described by Rougon et al. [36, 38]. Nonspecific binding was reduced by preincubating the antisera with the KLH carrier (1 mM). Bound antibodies were detected by reaction with ^{125}I-Prot A (*S. aureus* protein A) (10^6 cpm/ml).

Affinity Purification of Antibodies from Immunoblot

For affinity purification of anti-β_2-AR antibodies from the original sera, deoxycholate solubilized membranes (10 mg protein) were separated on 10% SDS-polyacrylamide gels with a 10-cm-wide sample slot and blotted onto nitrocellulose filters. A 0.5-cm strip was cut from each side of the filter and stained with the respective antisera. Using the labeled strip as a guide, the regions containing the interesting bands were separately exised, saturated 1 hr at 37°C with 5% PBS (phosphate buffered saline) milk as described [36, 38] and incubated overnight at 4°C in a 1 : 500 dilution of the respective antisera. After three 15-min washes in PBS, bound antibodies were eluted for 2 min in 2 ml of 0.2 M glycin-HCl buffer, pH 2.5. The solution was neutralized with 1 M Tris base, pH 8.0, and extensively dialyzed as previously described [18].

Enzymatic Linked Immunosorbent Assay (ELISA)

Cells were grown to confluence in 96-well culture plates in Dulbecco's modified Eagle medium (DMEM) with 10% fetal calf serum (FCS). Cells were rinsed, fixed with 1% paraformaldehyde then incubated with 1 : 250 diluted antisera (100 μl/well) in DMEM HEPES with 10% FCS for 12 hr at 4°C. Bound antibodies were detected using goat antirabbit antibodies labeled with peroxidase diluted 1 : 10,000. The colorimetric reaction was determined at 405 nm using a Titertek Multiskan.

Cell Culture

The rat C6 astrocytoma and human HT29 adenocarcinoma cell lines were maintained in DMEM supplemented with 10% FCS and antibiotics in 7% CO_2.

Immunofluorescence

Cells were dissociated from embryonic day 16 mouse brains, seeded on poly-L-lysine coated coverslips and kept for four days in vitro as described previously [37]. In some experiments, before incubation with the anti-64,000 dalton purified antibodies, cells were fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton X100 in PBS/albumin for 5 min. Bound antibodies were detected with rhodamin conjugated goat antirabbit IgG. In control experiments, preimmune sera or immune sera preincubated with the corresponding peptides were used.

Results

Characterization of the Antisera

Peptides were chosen according to their predicted membrane location based on the proposed model for hamster lung β_2-AR [9] and their hydrophilicity with a sufficient length to exhibit optimal immunogenicity (*see* Table). Antisera were generated in all eight rabbits (peptide 1: 1–4; peptide 2: 5–8). Figure 1 shows competitive displacement of ^{125}I-peptide by its unlabeled counterpart. Examination of the binding affinity by Scatchard plot analysis gave values around 10^{-8}, 10^{-9} M when expressed as dissociation constant values (K_d). In all subsequent studies, the antibodies have been used at a 1 : 500 dilution.

We then tested which of the antisera were able to recognize β_2-AR purified from hamster lung tissue, either under immunoblot or immunoprecipitation conditions. Figure 2*A* shows immunoprecipitation of ^{125}I-purified β_2-AR. For example, lanes *B* and *D* represent immunoprecipitation by antisera number 1 and 7 directed against peptide 1 and 2, respectively. Lanes *C* and *E* demonstrate that the immunoprecipitation could be blocked by adding 1 μM of the corresponding peptide in the incubation medium. Lane *F* shows the starting iodinated material.

Most of the antisera (1–4 directed against peptide 1 and 7 as well as 8 directed against peptide 2) were able to recognize purified β_2-AR under immunoblot condition as shown in Fig. 2*B*. Here again

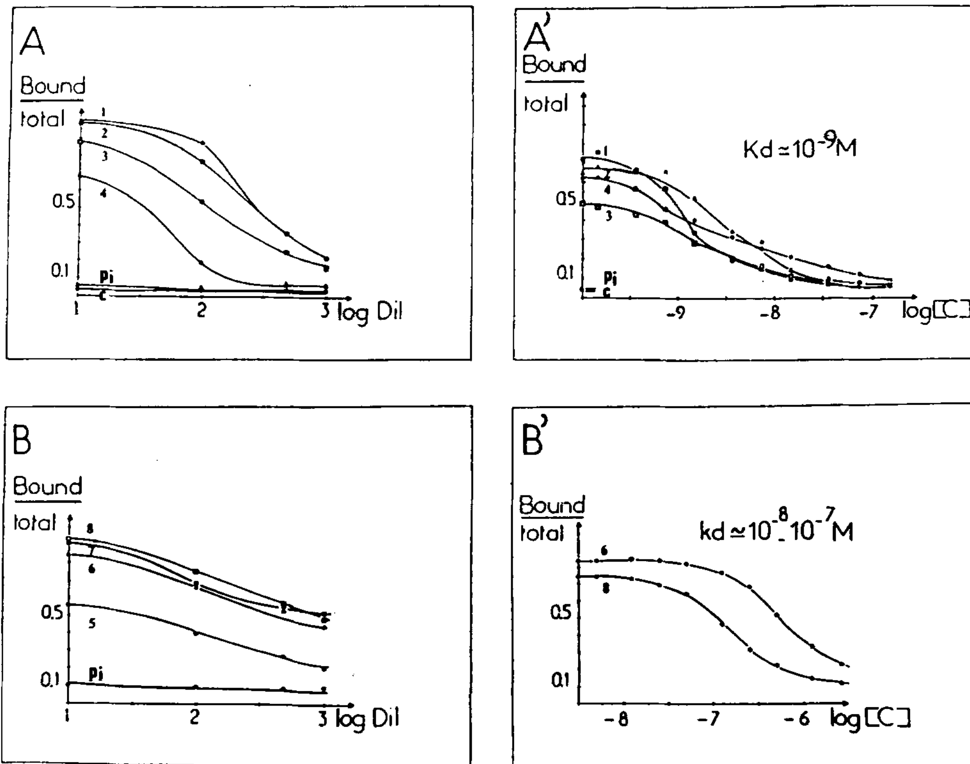


Fig. 1. Characterization of the antipeptide antisera. *A* and *B* show the binding of ^{125}I-labeled peptides to antisera (*A*: against peptide 1, *B*: against peptide 2) after the fourth injection. Serial dilutions were incubated in 0.5 ml PBS with 25,000 cpm of labeled peptide for 24 hr at 4°C. Bound was separated from free by precipitation with 25% polyethylene glycol. *A'* and *B'* demonstrate the inhibition of binding of ^{125}I-labeled peptide by the unlabeled homologous peptide. The immune complexes were formed in 0.5 ml PBS containing antisera diluted 1 : 500, unlabeled peptide (7.4×10^{-10}–3.7×10^{-6} M) and 25,000 cpm of ^{125}I-labeled peptide. Nonspecific binding determined from nonimmune (C) or preimmune serum (Pi) was ≈5%. Bound over total radioactivity was plotted *vs.* the log of the peptide concentration

excess (1 μM) of corresponding peptide was able to inhibit the binding, this is shown lanes *I* and *J* for antiserum 2 (against peptide 1) and lanes *K* and *L* for antiserum 7 (against peptide 2). Two antisera were also able to immunoprecipitate ^{125}I-cyanopindolol labeled β_2-AR as shown in Fig. 3. Various dilution of crude antisera were incubated with ^{125}I-cyanopindolol labeled pure β_2-AR. Both antisera 1 directed against peptide 1 and 7 directed against peptide 2 were able to immunoprecipitate around 13 to 15% of the counts in the assay. This immunoprecipitation was specific as it could be inhibited by excess (1 μM) of the corresponding peptide.

Antibodies Recognized β_2-AR or β_2-AR-Like Molecules in Hamster Tissues

Due to the low amount of β_2-AR expression in tissues, we prepared enriched fractions of membranes from hamster tissues. When they were submitted to immunoblot analysis, probed by our antisera directed against peptides 1 and 2, we could reveal immunoreactive bands. To determine conclusively that the reaction was occurring with molecules exhibiting the peptide sequence, we presaturated the antibodies with an excess of peptide (1 μM) and verified which bands were not further detected. Results are shown in Fig. 4 for hamster lung membranes with antisera 4 and 8 directed against peptide 1 and 2, respectively. We determined that the molecular weight (M_r) of the bands specifically recognized were 64,000, 57,000, 47,000 and 44,000 (±2000) daltons. These results were highly reproducible although the intensity of the bands varied slightly from one experiment to another. Occasionally, antisera against peptide 2 also revealed bands around 38,000 daltons. In order to test the relationship between the different bands sharing a related peptide sequence and the β_2-AR, we conducted the following experiments, whose results are shown in Fig. 5. First, to decide whether they could be degra-

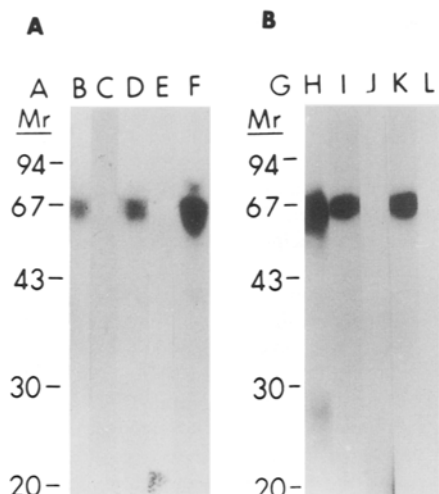


Fig. 2. Immunoblotting and Immunoprecipitation of purified hamster β_2-AR. (*A*) Shows immunoprecipitation of ^{125}I-purified hamster lung β_2-AR. Lane *A* shows the M_r standards. Lane *B* contains ≈7,000 cpm β_2-AR immunoprecipitated by antiserum 1. Lane *C* demonstrates complete blockade of immunoprecipitation by 1 μM peptide 1. Lane *D* contains ≈9,000 cpm ^{125}I-purified hamster lung β_2-AR immunoprecipitated by antiserum 7. Lane *E* shows complete blockage of immunoprecipitation by 1 μM peptide 2. Lane *F* shows the starting material, ≈50,000 cpm ^{125}I-purified hamster lung β_2-AR. Immunoprecipitation could not be blocked by 100 μM of noncorresponding peptide (*not shown*). (*B*) Shows a typical western immunoblot against purified hamster lung β_2-AR. Each lane contains 5 pmol of β_2-AR. Antisera were incubated at a dilution of 1:500 as discussed in Materials and Methods. Lane *G* shows the M_r standards. Lane *H* shows an aliquot (≈30,000 cpm) of chloramine T labeled ^{125}I-β_2-AR. Lanes *I* and *J* show immunoblot patterns obtained with antiserum 2 without and with 1 μM peptide 1. Lanes *K* and *L* show patterns obtained with antiserum 7 without and with peptide 2. Each antiserum recognizes a specific band of M_r ≈65,000 daltons blocked by the corresponding peptide. This band corresponds to the purified β_2-AR (lane *H*).

dative products occurring during membrane preparation, we mixed with hamster lung tissue 10^6 cpm of ^{125}I-labeled purified β_2-AR (Fig. 5, lane *A*). Although we conducted this preparation in presence of a cocktail of protease inhibitors (*see* Materials and Methods), it is clear that some degradation could occur as, beside the major 64,000-dalton band, bands migrating at 57,000, 44,000 and 25,000 daltons could be detected (Fig. 5, lane *B*).

To further assess the nature of the degradative processes and the identification of our immunoreactive products, we submitted pure iodinated β_2-AR to endoglycosidase F and/or mild tryptic degradation. Treatment with endoglycosidase F gave a major band at 44,000 daltons after 8 hr of treatment (Fig. 5, lane *E*), with an intermediate product migrating at 57,000 daltons (Fig. 5, lane *D*).

Mild trypsin treatment conducted on pure β_2-AR gave radioactive bands of 38,000, 32,000 and 25,000 daltons (Fig. 5, lanes *F* and *G*). When this treatment was applied to endoglycosidase F-treated product, the 57,000- and 44,000-dalton bands decreased and gave 38,000, 32,000 and 25,000 daltons (Fig. 5, lanes *H* and *I*). These data are consistent with those cited in the literature for β_2-AR [4, 11]. Thus, the 64,000-dalton species represents the glycosylated form of the β_2-AR. The lower molecular weight species are very likely degraded, oxidized or deglycosylated forms of the β_2-AR [11, 32, 40], while the 146,000-dalton species, which is not always detected, may be a polymer. The specificity of the antisera was further confirmed by failure to react with purified rhodopsin (Fig. 4, lane *H*), a receptor-related molecule [47], or with hamster heart, a β_1-AR-rich tissue [16], Fig. 4, lanes *F* and *G*.

We also immunopurified fractions of the antisera-recognizing molecules sharing related peptide sequence as described in Materials and Methods. We then check as a positive control the ability of purified antibodies to recognize purified β_2-AR under immunoblot conditions. This is shown in Fig. 6, lanes *B* and *C* for the antibodies directed against peptide 1 eluted from the 64,000-dalton band (not shown for antisera against peptide 2), meaning that these antibodies are directed against β_2-AR. Antibodies eluted from other bands (i.e., 57,000-, 47,000- and 44,000-dalton bands, respectively), were still able, on membrane extract from hamster lung, to recognize the band from which they were eluted. However, no cross-reaction could be seen with other bands as shown in Fig. 6, lane *D* for the 47,000-dalton species. These results are rather difficult to interpret as all these proteins share a similar peptide sequence. Nevertheless, we can postulate that even in immunoblot conditions, the peptide sequence included in a high molecular weight protein would maintain specific conformations only recognized by subpopulations of antibodies; the affinity of these antibodies could be too low to bind to the same sequence in a slightly different conformation.

Distribution of Immunoreactivity in Hamster Tissues

The β_2-AR is an ubiquitous molecule largely distributed in various tissues. To test the usefulness of our antisera in detecting such molecules, we set up an immunoblotting assay and examined their reactivity for membrane from different tissues (*not shown*). In liver, the major band detected migrated at 57,000 daltons. This band was also detected in lung and probably corresponded to a degradative product.

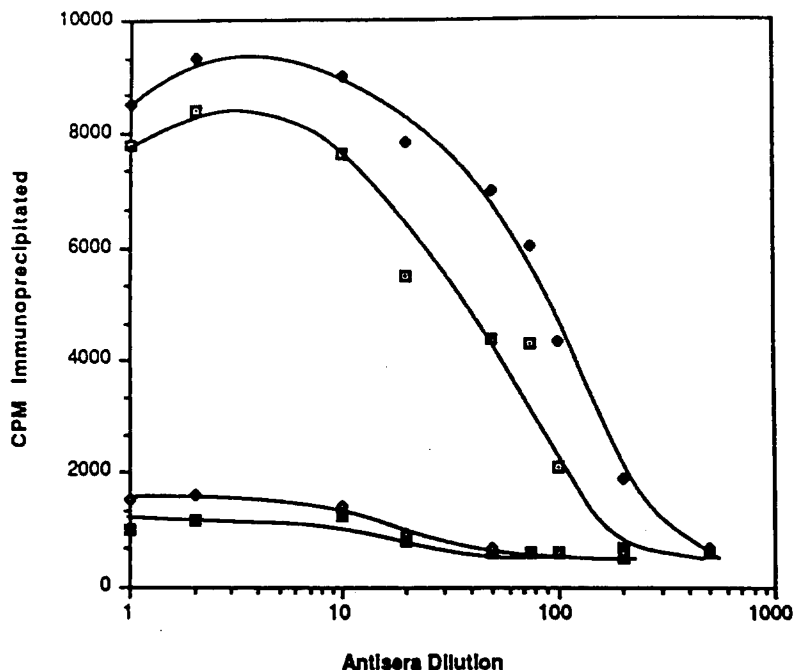


Fig. 3. Immunoprecipitation of ^{125}I-cyanopindolol labeled pure β_2-AR with various dilutions of crude antisera 1 and 7. Both antisera 1 and 7 (□, ◆) were able to immunoprecipitate 13–15% of the counts. Inhibition was made by 1 μM of corresponding peptide (■, ◇). Immunoprecipitation was not inhibited by 1 μM of noncorresponding peptide for either antisera. The curves were supimposable with those without competing peptide (*not shown*). Preimmune sera and Prot A-Sepharose in the absence of sera yielded curves superimposable with those of sera blocked with the respective corresponding peptide. Points shown represent the means of duplicates. The experiment was repeated twice with markedly identical results

This was also the case for cortex as well as embryonic brain. In heart (Fig. 4, lanes *F* and *G*), no specific band could be seen, this is in agreement with data reporting that βAR detected in this organ are of the β_1 subtype [16, 30].

Immunoblotting of C6 and HT29 Cell Extracts

Immunoblotting technique was also used to probe tissues from species other than hamster. For this purpose, the C6 rat astrocytoma cell line demonstrated by various investigators as exhibiting around 4,000–5,000 ^{125}I-cyanopindolol binding sites per cell [2] and the human adenocarcinoma HT29 [15, 27] cell line were utilized. Figure 7 demonstrates a typical immunoblot of deoxycholate-extracted membranes from these cell lines. Antisera against peptide 2 consistently revealed a band at 53,000 daltons in both species (Fig. 7, lanes *C* and *E–G*); however, no band at 64,000 daltons was seen. Antisera against peptide 1 demonstrated the same band in C6 cells (Fig. 7, lane *B*), but not in HT29 cells under our conditions. This band probably represents a degradation product, or different glycosylation state of the β_2-AR. It is likely that the 53,000-dalton band is truly the β_2-AR, since antisera against both epitopes specifically recognize the same band on immunoblot, and the sequence of the human and hamster β_2-AR are quite homologous in those regions (*see* Table) [11].

Location of the Recognized Sequences with Respect to the Lipid Bilayer

The extracellular location of the synthesized peptide domains was confirmed both by ELISA and immunofluorescence staining of live cells. The above results confirm that the antisera against peptides 1 and 2 are sensitive and specific for β_2-AR under denaturing conditions. In order to determine the orientation of the β_2-AR within the lipid bilayer of the cell membrane, it was also necessary that the antisera were immunoreactive with the native (non-denaturated) β_2-AR. Figure 8, panels *A* and *B*, show that a positive and quantifiable signal was obtained when antisera were reacted with rat C6 astrocytoma cells and human HT29 adenocarcinoma cells. The signal obtained with preimmune sera was subtracted from the values given in Figure 8. This finding suggests that peptide domains 1 and 2 of the β_2-AR are extracellularly located.

Biochemical and autoradiographic techniques have localized β_2-AR to various regions of the central nervous system [6]. However, there are few reports of cells expressing such receptors. To that purpose antibodies micropurified from the 64,000-dalton band, were used to identify cultured neural

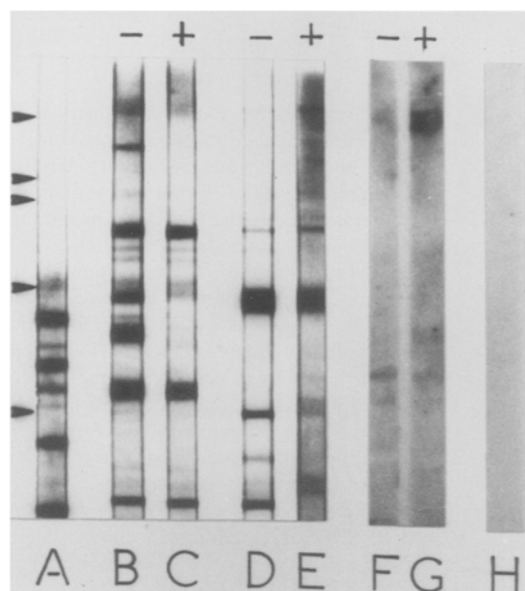


Fig. 4. Immunoblotting of hamster lung and heart membrane extracts and pure rhodopsin. After electrophoresis on a 10% SDS-polyacrylamide gel and transfer to nitrocellulose sheets, hamster lung membrane-extracted proteins were reacted directly with the antiserum 4 (1:500) against peptide 1 (lances *B* and *C*) or the antiserum 8 against peptide 2 (lanes *D* and *E*). Lane *A* is a control with preimmune sera. After reaction with the antisera, the protein bands were visualized by incubation with ^{125}I-Prot A. Lanes *C* and *E* were blocked by preincubation with 1 μM of corresponding peptide. Bands at 146,000, 64,000, 57,000 and 47,000 daltons (lane *C*) and 64,000, 44,000, 38,000 and 33,000 daltons (lane *E*) disappeared indicating they exhibit the corresponding sequences. This experiment is representative of numerous similar experiments. Lanes *F* and *G* correspond respectively to an immunoblotting assay done with antiserum 2 presaturated or not with its corresponding peptide (peptide 1) on hamster heart membrane extract. No band disappeared after presaturation, which is in agreement with the fact that this organ expressed mainly β_1-AR subtype. Lane *H:* antibodies directed against peptide 1 (or peptide 2: *not shown*) do not reveal pure rhodopsin

cells that express β_2-AR-like immunoreactive molecules. Figures 9 and 10 show immunofluorescence in cell cultures prepared from embryonic day 16 mouse brain and maintained in culture for four days. Both sets of antisera reacted specifically with the same population of cells. These cells appear to be neurones, or possibly type II astrocytes. The staining pattern of the neuron-like cells was not uniform, and only 10% of the cell population was labeled. These labeled cells were found in clusters, possibly derived from the same precursor and sharing a common phenotype. Fibroblasts and type I astrocytes were labeled weakly or not at all (Fig. 9*A–D*). The fact that nonpermeabilized live cells (Fig. 10) stained with equal intensity to permeabilized or fixed cells (Fig. 9) confirms the extracellular location of the peptide domains. The specificity of the staining was ascertained by utilizing preimmune sera as controls (Fig. 9*E–F*; Fig. 10*C–D*).

Discussion

Dohlman et al. [11] have proposed a model in which the β_2-AR resides in the membrane in a conformation, which crosses the lipid bilayer seven times, with an extracellular amino-terminus containing two potential glycosylation sites and an intracellular carboxyl-terminus. Through various biochemical techniques, they were able to confirm this conformation for transmembrane segments IV–VII. Additionally, they suggested that the amino-terminus was oriented extracellularly based on endogylcosidase F treatment of tryptic fragments of purified hamster β_2-AR.

In this report, we utilized antibodies raised to specific peptide sequences in the domains near the amino-terminus and in the putative second extracellular loop to confirm their extracellular location.

The fact that these antipeptide antibodies can interact with β_2-AR is demonstrated by their ability to immunoblot and immunoprecipitate purified β_2-AR, and to immunoblot specific bands in hamster lung membranes under reducing conditions, which correspond to the known M_r for native (64,000 daltons), partially (57,000 and 47,000 daltons) and completely (44,000 daltons) deglycosylated, or tryptic degraded (38,000 daltons) β_2-AR [4, 11]. Surprisingly, the antibodies able to recognize the 64,000 species in immunoblot conditions did not recognize very well species of lower molecular weight, and conversely. We can consider that the size of the peptides used as immunogens is large enough to induce several populations of antibodies with different specificities. Then it is likely that some populations of antibodies would preferentially recognize their epitopes according to their conformation or environment. This might be especially true when the sequence is included into a lower molecular species in which one of the terminal extremities of the antigenic sequence is free. We cannot exclude that these lower M_r species are β_2-AR unrelated molecules; however, they reacted with antisera directed against sequences shared by β_2-AR and unglycosylated or trunkated β_2-AR can be differently recognized as epitopes could be unmasked or better exposed. Accordingly, the intensity of the bands detected might not reflect the quantity of protein

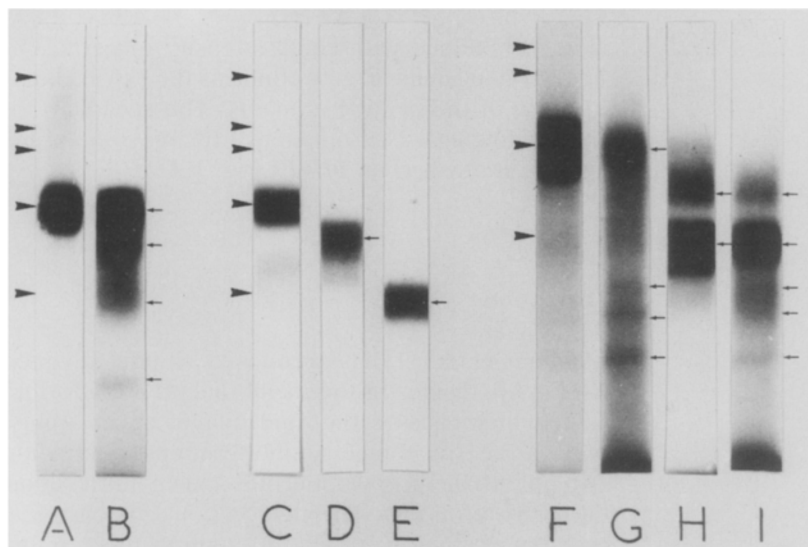


Fig. 5. Enzymatic degradative products of β_2-AR. Pure iodinated receptor (lane *A*) was mixed with hamster lung tissue and submitted to a complete cycle of membrane preparation; then radioactive products were examined on a 10% SDS-polyacrylamide gel (lane *B*). Iodinated receptor (lane *C*) was submitted to endoglycosidase F treatment under conditions described in Materials and Methods for 4 and 8 hr, respectively (lanes *D* and *E*). Iodinated receptor (lane *F*) was submitted directly to mild tryptic digestion (lane *G*) or for 6 hr to endoglycosidase F treatment (lane *H*) followed by mild tryptic digestion (lane *I*) as described in Materials and Methods. Radioactive products were examined on a 12% SDS polyacrylamide gel

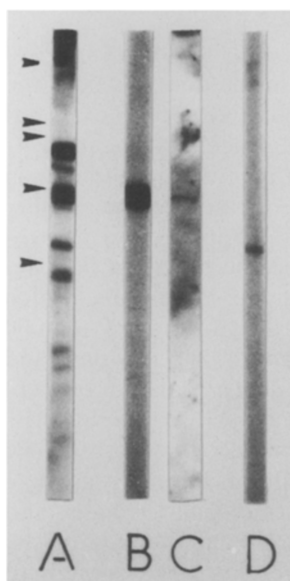


Fig. 6. Immunopurification of antibodies and antigenic relationship between different species recognized by the antisera and β_2-AR. Antibodies were immunopurified as described in Materials and Methods; they were diluted 1 : 10 in 5% PBS milk and incubated on 500 μg hamster lung membranes blotted onto nitrocellulose sheets. Lane *A*: antisera directed against peptide 1 as well as peptide 2 (*not shown*) recognized proteins migrating at 64,000, 57,000, 47,000 and 44,000 daltons. To assess the relationship between these M_r species, antibodies were eluted from each band and incubated with other lung membrane extracts. Lanes *B* and *D* demonstrate that the 64,000- and 47,000-dalton species eluted antibodies were still able to recognize molecular species from which they have been eluted, meaning that the elution process had not abolished their activity. Lane *C* demonstrates that antibodies eluted from the 64,000-dalton species are able to reveal pure β_2-AR blotted onto nitrocellulose sheets

but rather the affinity of the antiserum for a given conformation of the antigen.

It is noteworthy that the two sets of antisera directed against peptides 1 and 2, respectively, essentially bind to similar M_r species in hamster lung tissues as well as in human or rat cell lines; thus, if the detected molecules were not true β_2-AR, they will be closely similar according to their sequences in the probed regions. The 53,000-dalton species detected in the rat C6 and human HT29 cell lines correspond to the M_r reported for the β_2-AR for another human cell line, the A431 [19, 30]. These cell lines are all of tumoral origin. The different M_r may reflect a metabolic perturbation such as modification of the glycosylation state of the molecules.

The fact that some of our antibodies are able to interact with the labeled complex ^{125}I-cyanopindolol-β_2-AR indicates thay they do not recognize the ^{125}I-cyanopindolol binding domain of the β_2-AR. This result is in agreement with the literature considering that membrane spanning domains are crucially involved in the binding of adrenergic ligands [24, 48].

ELISA, as well as immunofluorescence results, showed that antibodies against the two chosen sequences were also reacting with native forms of the molecules and with live cells; this is proof that these sequences are exposed on the external part of the cell membrane. This provides further evidence for the correctness of the proposed model depicting the arrangement of β_2-AR in the membranes [9, 10]. The central nervous system has been shown to have β_2-AR in various regions by autoradiographic and biochemical techniques [6]. This study demon-

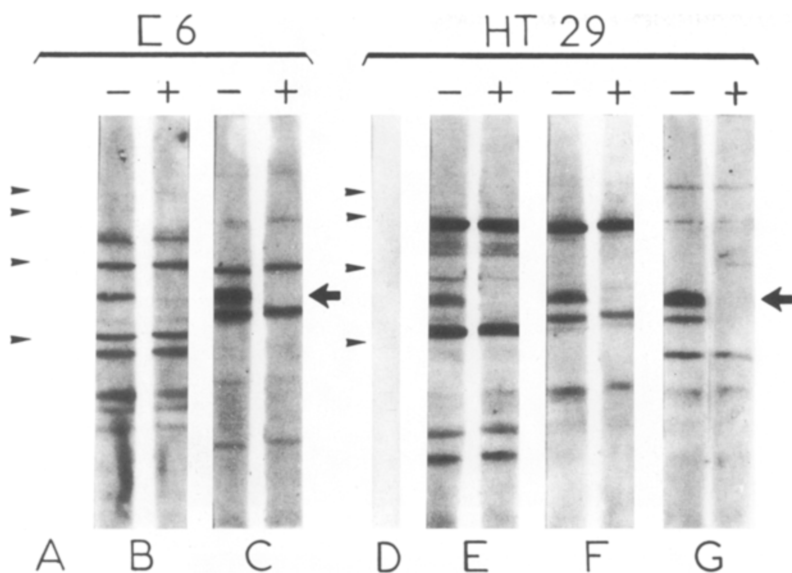
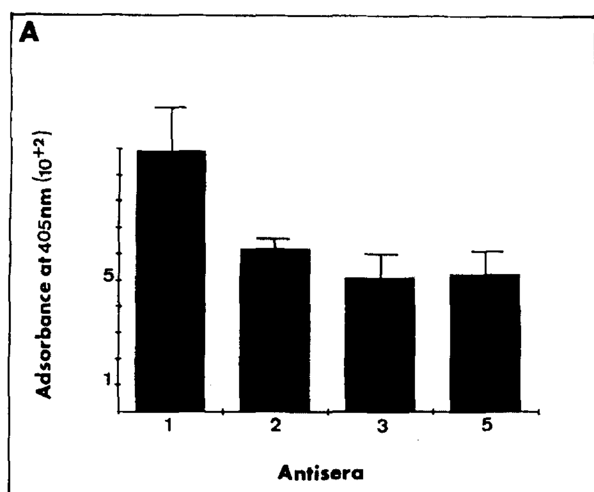


Fig. 7. Immunoblotting of HT29 and C6 cell line membrane extracts. Experiments were conducted as in Fig. 4. The left panel shows immunoreactive bands detected in the rat C6 astrocytoma cell line. Lane *A* represents preimmune serum control. Lanes *B* and *C* demonstrate the results from incubation of antisera directed against peptide 1 and 2, respectively. In both cases, preincubation with the corresponding peptide (lanes marked +) blocked the 53,000-dalton band. The right panel shows the immunoreactive bands detected in the human HT29 adenocarcinoma cell line. Lane *D* represents the preimmune control. Experiments with three different antisera against peptide 2 are shown (lanes *E*, *F* and *G*). In all cases, the 53,000-dalton band was blocked. Under the immunoblot conditions, the HT29 cell line was not immunoreactive with antisera against peptide 1



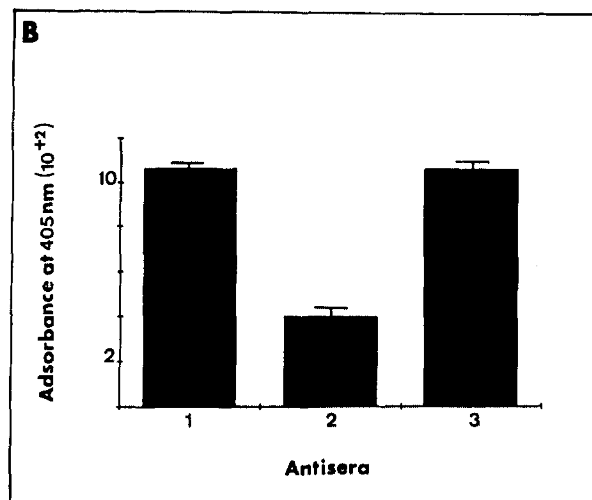


Fig. 8. Antipeptide antisera to β_2-AR reacted with determinants located on the extracellular surface. (*A*) Rat C6 astrocytoma cells and (*B*) human HT29 adenocarcinoma cells were plated in 96-well Costar culture plates at a density of 10^4 cells/well. Before incubation with antisera, cells were washed with culture medium without FCS, and slightly fixed with 1% paraformaldehyde at room temperature. This was to prevent internalization of the bound antibody. Cells were then incubated at 4°C for 12 hr with antisera (1 : 250). The plates were then washed and incubated with peroxydase-labeled goat antirabbit antisera for 2 hr at room temperature. The reaction was developed with 2, 2′-azinobis (3-ethylbenzothiazoline sulfonic acid) and intensity measured at 405 nm. Adsorbance is shown with (*A*) antisera against peptide 1 (1–3) and 2 (5) for C6 cells, and (*B*) with antisera (1–3) against peptide 1 for HT29 cells. Control values obtained with preimmune sera were subtracted from each condition

strates the power of these antisera as probes to detect live cells bearing immunoreactive β_2-AR and to analyze the expression of the β_2-AR independently of its ligand binding activity. The staining pattern observed is very similar to data published by Ventimiglia et al. [46] using an anti-idiotypic polyclonal antibody against β_2-AR. However, there is a difference in that in our culture conditions, astrocyte type I were only weakly or not labeled. As an explanation, it is known that the level of receptor expression by astrocytes is highly variable, with a general trend towards an increase in receptor expression with time in culture [44] or during the cell cycle [7]. Thus β_2-AR expression may be a property of terminally differentiated cells in the central nervous system.

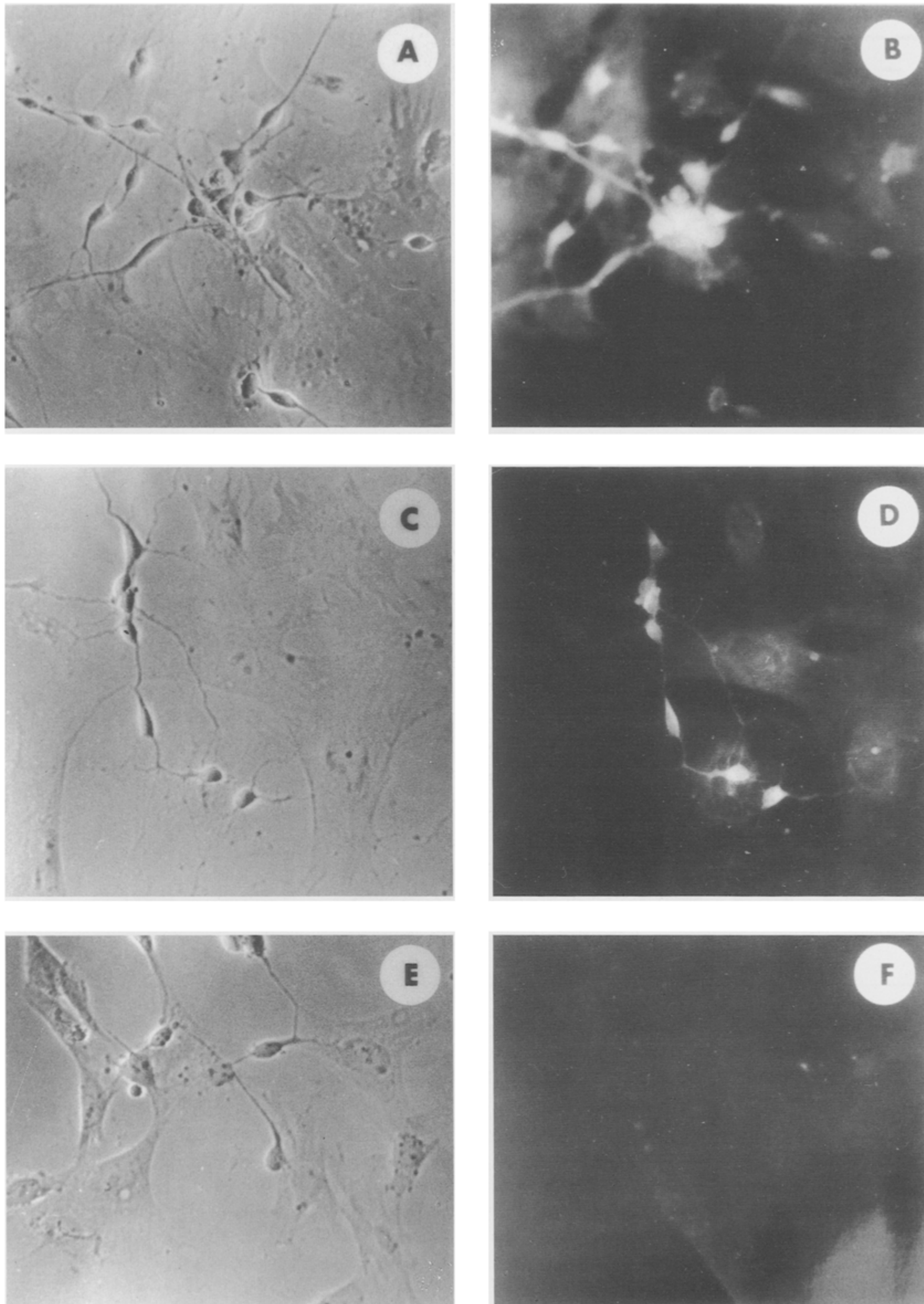


Fig. 9. Indirect immunofluorescence labeling of fixed mouse brain primary cultures. Primary cultures were prepared from embryonic mouse brain harvested at day 16, grown on poly-L-lysine coated coverslips for four days. (*A–D*) Cells have been fixed with 2% paraformaldehyde and permeabilized with 0.1% Triton X100 PBS/albumin. Fixation procedure avoids losing cells during washing steps (magnification 520×). *E* and *F* show control done with preimmune serum. (*B*) Staining with antipeptide 1 antisera micropurified on the 64,000-dalton band; (*D*) with antipeptide 2 antisera purified in the same way; and (*E*) with a preimmune serum (all at 1 : 50). (*A*, *C* and *E*) The phase contrast micrographs corresponding to *B*, *D* and *F*, respectively (magnification 520×)

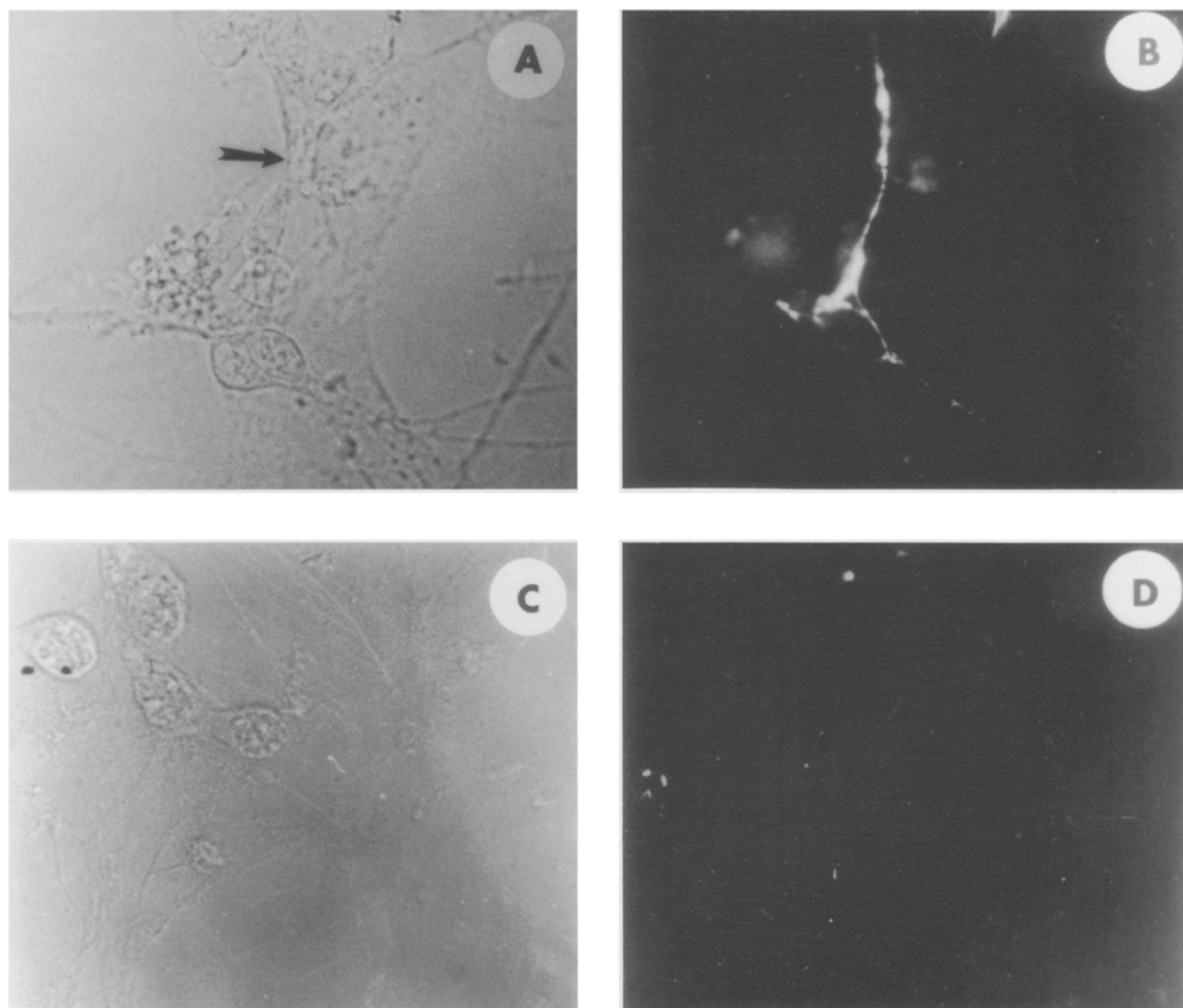


Fig. 10. Indirect immunofluorescence of live cell primary culture. Cells were cultured as in Fig. 9. Live cells were incubated with either a 1 : 50 dilution of a mixture of micropurified antisera against both peptides (*A*, *B*) or (*C*, *D*) preimmune serum. (*B*) Labeled cells are clustered, and neurites are brightly labeled (*A*) (arrow). The fact that live cells could be stained indicates that the recognized epitopes are extracellularly located. (*A* and *C*) Phase contrast micrographs corresponding to *B* and *D*, respectively (magnification 768×)

The fact that in these experiments the two sets of antisera are exhibiting the same pattern of staining according to the phenotype of labeled cells is in favor, but not proof that they bind to the same molecules. The percentage of immunoreactive neuronal cells was found around 10%. This might indicate that a specific population of brain neurones may selectively express β_2-AR. In culture, this expression may be influenced by diverse and undefined environmental factors.

In conclusion, micropurified antipeptide antibodies raised against two putative extracellular domains of the β_2-AR were utilized in live cells to confirm the extracellular nature of these epitopes with respect to the cell membrane. This approach should yield valuable information about the structure-function relationship in native receptor, such as defining domains for signal transduction or ligand binding. Such tools should prove a valuable adjunct to mutagenesis techniques.

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